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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 14 March 1996 in connection with Application No. PN 8703 for a patent by THE AUSTRALIAN NATIONAL UNIVERSITY filed on 14 March 1996.

I further certify that the annexed specification is not, as yet, open to public inspection.

WITNESS my hand this Nineteenth day of March 1997

DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

Regulation 3.2

The Australian National University

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION
for the invention entitled:

"Treatment of auto-immune insulin-dependent diabetes mellitus"

The invention is described in the following statement:

TREATMENT OF AUTO-IMMUNE INSULIN-DEPENDENT DIABETES MELLITUS

FIELD OF THE INVENTION

This invention relates to the use of *Coxiella burnetti* antigen(s), and in particular killed *Coxiella burnetti* in the form of Q fever complement fixing antigen phase I (QFA) or Q fever vaccine (QVAX) as treatment for the inhibition or prevention of insulin-dependent diabetes, IDDM, and treatment for the inhibition or prevention of pancreatic beta-cell destruction in islet tissue transplantation recipients.

BACKGROUND TO THE INVENTION

Diabetes mellitus is defined by the presence of hyperglycemia. During the 1960s insulin-dependent diabetes, IDDM, or type 1, was distinguished from non-insulin dependent diabetes, NIDDM, or type 2 diabetes. During the 1970s evidence was uncovered suggesting the autoimmune nature of type 1 diabetes, in which the patient's own immune system destroys the cells responsible for insulin production, the beta cells residing in the islets of Langerhans of the pancreas. IDDM in humans and in the non-obese diabetic mouse (NOD mouse) and the BB rat is an autoimmune disease associated with the development of auto-antibodies reactive to islet-associated antigens (Atkinson *et al.*, 1986). Susceptibility to the development of disease, both in humans and in animals, is under genetic control. Genes of the major histocompatibility complex (MHC), specifically those found in the class II MHC region, which control structures involved in presentation of antigen to the cellular components of the immune system, are to a large extent responsible for the disease-prone status (Davis *et al.*, 1989).

Diabetes in both humans and NOD mice, however, is not a purely genetic disease. Thus, in the case of identical twins, there is a relatively low concordance (of the order of 35-40%) for the development of disease. There is

also a low concordance for the development of disease in the disease-prone animal models, such as NOD mice, where individuals are genetically identical (Pozzilli *et al.*, 1993).

Environmental factors contribute to the development of disease. The expression of disease in animal models is known to be negatively correlated with exposure to infectious agents, thus, the incidence of diabetes is greater in cleaner environments (Wilberz *et al.*, 1991; Like *et al.*, 1991). This was an unexpected finding as one hypothesis for the development of autoimmune disease involves the notion that the disease might be precipitated by molecular mimicry, and that particular infections may provide the trigger that initiates the disease process in the first place. The inverse of this, however, appears to be the case with the disease incidence being considerably higher in specific pathogen-free environments (Wilberz *et al.*, 1991). Disease incidence in humans varies greatly between countries (Group, 1988); these differences are not attributable only to ethnic (and thus genetic) differences since studies have shown that migrants from countries with a low incidence who migrate to countries with a high incidence of IDDM will have an increased chance of developing the disease over those remaining in the country of origin (Patrick *et al.*, 1989). The incidence of disease is also known to be lower in countries having less sophisticated public health systems (Karvonen *et al.*,). Migration studies further suggest that environmental factors are responsible for, at least in part, the lower disease incidence (Karvonen *et al.*,) in less developed countries.

In animal models, a single immunostimulation with live, infectious BCG (*Mycobacterium bovis*; Bacille calmette-guérin) vaccination (Yagi *et al.*, 1991) or powerful immuno-adjuvants based on heat-killed *M. tuberculosis* (or *M. butyricum*) either early or late in the disease process, can largely suppress the development or progression of disease respectively (reviewed in Bach, 1994). Freund's complete adjuvant (FCA) (often referred to as complete Freund's adjuvant or CFA), which consists of a suspension of heat-killed *M. tuberculosis*

(or *M. butyricum*) in mineral oil together with a surfactant is considered to be one of the most powerful immunological adjuvants. Immunostimulation with a single injection of FCA has been shown to substantially protect both NOD mice (Sadelain *et al.*, 1990a; McInerney *et al.*, 1991) and BB rats (Sadelain *et al.*, 1990b) from developing diabetes. Immuno-adjuvants such as bacterial lipopolysaccharide (LPS) or muramyl dipeptide (MDP) which are not based upon either live or heat-killed *Mycobacterium sp.* are not effective in preventing diabetes. Another biological response modifier, OK-432, prepared from streptococci, when administered once weekly to NOD mice, has been shown to prevent the onset of diabetes until mice reach 24 weeks of age (Toyota *et al.*, 1986); no information exists showing whether or not these animals remain free of diabetes once the therapy is stopped. It has also recently been shown that the immunomodulating drug quinoline-3-carboxamide (Linomide) when given continuously in the drinking water to NOD mice, starting at a young age (5 weeks), will block the onset of diabetes until the mice are at least 40 weeks old (Gross *et al.*, 1994). This agent is not, however, as effective when therapy is started when the mice are 16 weeks old; in this case the cumulative incidence of diabetes is 28% by the time the mice are 42 weeks old even though the drug is given on a continuous basis (Gross *et al.*, 1994).

FCA appears to induce a cell-mediated protection against development of diabetes. It has, for example, been shown that spleen cells taken from NOD mice, which had been treated with FCA, were capable of suppressing the response of co-cultured control syngeneic spleen cells to mitogens (Sadelain *et al.*, 1990a; McInerney *et al.*, 1991). When spleen cells are taken from BCG vaccinated NOD mice and passaged into naive NOD mice, the latter are protected from developing diabetes (Harada *et al.*, 1990; Yagi *et al.*, 1991). The same effect has been observed in FCA-treated BB rats (Qin *et al.*, 1992). It should be noted that others have found FCA to be less effective than BCG in transferring protection in this manner (Qin *et al.*, 1993; Ulaeto *et al.*, 1992).

Recurrence of disease in grafted tissue is a major factor which interferes with the transplantation of pancreatic islets into spontaneously diabetic NOD mice. Currently it is not possible to successfully transplant islets into animals that have developed disease spontaneously without recourse to extensive immunosuppression (Wang *et al.*, 1991). Recurrence of disease can, however, be blocked in spontaneously diabetic NOD mice if they are treated with FCA (Wang *et al.*, 1992; Lakey *et al.*, 1992) or BCG (Lakey *et al.*, 1994) prior to transplantation.

Immunostimulation of NOD mice by either BCG or FCA does not totally block the autoimmune response, instead the response is converted from a destructive into a non-destructive form of auto-immunity (Shehadeh *et al.*, 1993). Thus, functionally these agents do not prevent or reverse autoimmunity directed against islet tissue, but, they do prevent development of insulin-dependent diabetes.

It would, therefore, appear that deviation of the immune response away from destructive autoimmunity by immunostimulation could offer a benign approach to the prevention of diabetes and the development of more effective means for the transplantation of islets in the case of existing disease. There is good evidence that immunostimulation, by giving a single immunisation with BCG at the time of diagnosis, can modify the natural history of the disease in humans (Shehadeh *et al.*, 1994).

Unfortunately, neither FCA nor BCG are ideal therapeutic agents for preventing the development of IDDM. Despite the fact that FCA is a very powerful immuno-adjuvant, it has not found wide-spread use outside the laboratory because of the adverse tissue reaction (severe ulceration) it provokes in mammals. In most countries FCA is banned from veterinary use and, of course, it cannot be used in humans. BCG is an infectious agent which cannot be given to certain individuals, especially those who are immunosuppressed. What is now required for blocking the development of IDDM in humans is the

development of agents that are sufficiently benign for general use and most effective in terms of deviating the immune response away from destructive autoimmunity. The use of non-infectious agents for such procedures offers a major advantage in clinical terms. Infectious agents, even those such as BCG, can be a problem if administered to an immunocompromised individual and, while FCA might be effective in humans, its use is precluded by the severe ulcerative skin lesions which develop following its use. An ideal agent would be capable of blocking the induction of diabetes as well as inhibiting disease recurrence in transplanted islet tissue in diabetic patients.

SUMMARY OF THE INVENTION

In work leading to the present invention, it has been discovered that Q fever (*Coxiella burnetti*) antigen(s), and in particular killed *Coxiella burnetti* in the form of either Q fever complement fixing antigen phase I (QFA) or Q fever vaccine (QVAX), when injected into NOD mice will inhibit diabetes in a high percentage ($\geq$90%) of treated mice. This agent is effective when given by either a single intraperitoneal or single subcutaneous injection. Several different doses have been tested and found to be effective. This agent is neither an infectious organism nor a recognised immuno-adjuvant, and yet, it has been discovered that it is as effective as either BCG or FCA in preventing the development of diabetes in NOD mice. It is effective when given as a single treatment, therefore, it has an advantage over other immune response modifiers such as OK-432 (Toyota *et al.*, 1986) and Linomide (Gross *et al.*, 1994) which must be administered in multiple treatment regimes to be effective.

In one aspect, the present invention provides a method for inhibition or prevention of the development of IDDM in a susceptible mammalian patient, which comprises administering to said patient an effective amount of Q fever antigen(s).

In another aspect, the present invention provides a method for inhibition or prevention of the progression of IDDM in a mammalian patient having IDDM,

which comprises administering to said patient an effective amount of Q fever antigen(s).

In yet another aspect, the present invention provides a method of treatment of a mammalian patient, in particular a human patient, having IDDM in order to prolong the survival of islet tissue transplanted into said patient, which comprises administering to said patient, either prior to or concurrently with transplantation of said islet tissue, an effective amount of Q fever antigen(s).

In a further aspect, the present invention provides a therapeutic composition for use in a method of treatment as broadly outlined above, which comprises Q fever antigen(s), together with at least one pharmaceutically acceptable carrier or diluent.

Finally, the present invention relates to the use of Q fever antigen(s) in the preparation of a therapeutic composition for use in a method of treatment as broadly outlined above.

Preferably, the mammalian patient is a human.

In a preferred embodiment of the present invention the patient is known to be likely to develop IDDM or is a patient who has been recently diagnosed to be in the early stages of IDDM or is a patient suffering from IDDM who will be transplanted with islet tissue from a non-diabetic donor.

The Q fever antigen(s) which are administered in accordance with the present invention include, in particular, killed *Coxiella burnetti* which may be provided in the form of either Q fever complement fixing antigen phase I (QFA) or Q fever vaccine (QVAX).

The active component is administered in therapeutically effective amounts. A therapeutically effective amount means that amount necessary at

least partly to attain the desired effect, or to inhibit or delay the onset of, inhibit the progression of, or halt or prevent altogether, the onset or progression of the particular condition being treated. Such amounts will depend, of course, on the particular condition being treated, the severity of the condition and individual patient parameters including age, physical condition, size, weight and concurrent treatment. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgement. It will be understood by those of ordinary skill in the art, however, that a lower dose or tolerable dose may be administered for medical reasons, psychological reasons or for virtually any other reasons.

The formulation of such therapeutic compositions is well known to persons skilled in this field. Suitable pharmaceutically acceptable carriers and/or diluents include any and all conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art, and it is described, by way of example, in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, USA. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the pharmaceutical compositions of the present invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the human subjects to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier and/or diluent. The specifications for the novel dosage unit forms of the invention are dictated by

and directly dependent on (a) the unique characteristics of the active ingredient and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active ingredient for the particular treatment.

Throughout this specification unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that Q fever antigen(s) and in particular killed *Coxiella burnetti* is more efficacious than either FCA or BCG in protecting beta cells from autoimmune destruction in NOD mice. Thus, NOD mice were treated with doses of either FCA, BCG or Q fever antigen(s), that were effective in preventing IDDM and their pancreases were examined when the mice reached 300 days of age. Significantly more viable beta cells were found in the pancreases of mice which had been treated with Q fever antigen(s) than in the mice treated with either FCA or BCG.

NOD female mice between 120 and 180 days of age, which have become diabetic spontaneously, treated with Q fever antigen(s) and in particular killed *Coxiella burnetti* and then transplanted with syngeneic islet tissue essentially as described in Bowen et al. (Bowen *et al.*, 1980) will maintain normal blood glucose levels, for example for at least one year. Control mice, treated with saline instead of Q fever antigen(s), and then similarly transplanted with syngeneic islet tissue, will all become diabetic, for example within two weeks of tissue transplantation. Histological sections of transplanted tissue taken from the saline treated group, at the time they become diabetic, will show a massive inflammatory infiltrate into the transplanted tissue, with essentially complete beta cell destruction. Histological sections taken for example one year after treatment

from the animals treated with Q fever antigen(s) will show little or no inflammatory infiltrate and the beta cells will be intact.

A variety of routes for administration of Q fever antigen(s) in accordance with this invention are available. The particular mode selected will depend, of course, upon the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practised using any mode of administration that is medically acceptable, meaning any mode that produces therapeutic levels of the active component of the invention without causing clinically unacceptable adverse effects. Such modes of administration include in particular parenteral (e.g. subcutaneous, intramuscular and intravenous) routes.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing the active component into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active component into association with a liquid carrier.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active component which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in polyethylene glycol and lactic acid. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed

including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Further features of the present invention are more fully described in the following Example(s). It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

EXAMPLES

In the following examples all specific pathogen-free NOD mice were obtained from the Animal Breeding Establishment, The Australian National University. Mice were housed under specific pathogen-free conditions with sterilised micro-isolator cages and bedding. Sterilised food and water were provided ad libitum. Cages were changed in laminar flow hoods using sterile technique by a laboratory assistant who was dedicated solely to this task and who was not responsible for the maintenance of any other animals. Cages were housed in hepa filtered ventilation racks with a 12 hour day night cycle. Routine serological monitoring was performed by the Murine Virus Monitoring Service, Primary Industries (Adelaide, South Australia), for evidence of infection by any of 15 different murine viruses. Since the implementation of this procedure in 1994, the NOD mouse colony used for the following examples has tested negative for all of these viruses.

Example 1:

This example demonstrates the effectiveness of a single 200 μl dose of Q fever complement fixing antigen Phase I (QFA) in inhibiting the occurrence of IDDM in NOD mice.

Female NOD littermates between 64 and 69 days of age were divided into two groups and injected intraperitoneally (ip) with either 200 μl of Q fever complement fixing antigen phase I (obtained from CSL, Melbourne Australia) or 200 μl of saline solution. Fifteen mice were injected with QFA while 14 littermates received saline solution. One animal from each group was found dead of unknown causes during the course of the experiment.

For comparison, in a similar manner, 17 female NOD littermates between the ages of 64 and 85 days of age were injected in the right rear footpad with 50 μl complete Freund's adjuvant (FCA) (Bacto lot # 784560) given as an emulsion in an equal volume of normal saline; 16 littermates received saline solution only.

Mice in all of the four groups were checked three times per week (Mon., Wed. and Fri.) for elevated glucose concentrations in their urine (Tes-Tape, Eli Lilly and Co, Indianapolis, IN). When mice were found to have raised urinary glucose levels, whole blood glucose levels were determined with a Companion 2 Sensor (Medisense, Cambridge, Mass). Where two consecutive daily blood glucose readings above the 95% confidence interval for NOD mice in this colony (3.9-9.1 mmol/L) were obtained, the animal was deemed to be diabetic. Animals were monitored until they reached 300 days of age or became diabetic. The results of this experiment are shown in Figure 1.

As indicated in Figure 1, at 300 days of age, 2 out of 17 mice (12%) in the FCA treated group had become diabetic while 12 out of 16 mice (75%) of their saline treated littermates became diabetic. At 300 days of age only 1 out of 14 (7%) of the QFA treated mice had developed diabetes compared to 8 out of 13 (62%) of their saline treated littermates.

Exampl 2:

This example demonstrates the effectiveness of single 20 or 70 μl doses of QFA in inhibiting the occurrence of IDDM in NOD mice.

Female NOD mice between 62 and 72 days of age were injected intraperitoneally (ip) with either 70 μl of QFA or 20 μl of QFA. Fifteen mice were injected with 70 μl of QFA and 15 mice received 20 μl QFA. One animal from the 20 μl group was found dead of unknown causes during the course of the experiment. The 200 μl saline injected group of female NOD mice from Example 1 served as control animals. Mice were checked three times a week for elevated glucose levels in their urine (Tes-Tape, Eli Lilly and Co, Indianapolis, IN.). When positive for urinary glucose, whole blood glucose levels were determined with a Companion 2 sensor (Medisense, Cambridge, Mass). Where two consecutive daily blood glucose readings above the 95% confidence interval for NOD mice in this colony (3.9-9.1 mmol/L) were obtained, the animal was deemed to be diabetic. The experiment was continued until the animals reached 260 days of age or became diabetic. The results of this experiment are shown in Figure 2. At 260 days of age only 2 out of 15 (13%) of the group treated with 70 μl of QFA and 1 out of 14 (7%) of the 20 μl QFA treated group had become diabetic, compared to 8 out of 13 (62%) of the saline treated NOD mice from Example 1 that served as controls for this experiment.

Example 3:

This example demonstrates that QFA is more efficacious than either FCA or BCG in protecting beta cells from autoimmune destruction in NOD mice.

A group of 10 specific pathogen-free, 61- 66 day old female NOD mice were injected in both rear footpads with 40 μl (total dose 80 μl) reconstituted freeze-dried live BCG (Pasteur Merieux, Lyon, France). A second group of 17, 64-85 day old female NOD animals, were injected with FCA emulsified with an equal volume of normal saline (50 μl in right hind footpad). A third group of 14

age-matched female NOD mice were injected, ip, with 200 µl of QFA. Two additional groups of 14 age-matched female NOD mice received either 70 or 20 µl of QFA, ip. When the mice had reached 300 days of age, 10, 13 and 11 mice from the BCG, FCA and each of the QFA treated groups respectively (all nondiabetic) were sacrificed by cervical dislocation and their pancreases removed for histological sectioning and examination. Thirteen age-matched nondiabetic female NODs were also sacrificed and their pancreases similarly examined. Pancreases were fixed in 10% neutral buffered formalin for 18 hours. Immunohistochemistry was used to stain for the presence of insulin and glucagon positive islets (DAKO Corporation, Carpinteria, CA). Serial 5 µm sections were stained for either insulin or glucagon and examined by light microscopy. As shown in Table 1 only 16% of islets in the BCG treated group stained positive for insulin production, 52% of islets in the FCA treated group stained positive for insulin and in the QFA treated group 87%, 77% and 75% of islets stained positive for insulin production in the 200, 70 and 20 µl treatment groups respectively. Seventy-three percent of the islets examined from pancreases of age-matched female NOD mice had insulin positive islets while 27% of the islets were positive for glucagon only.

TABLE 1

Treatment	% Insulin positive islets*	% Collapsed Islets * (Glucagon only)
BCG	**16** (11/69)	**84** (58/69)
FCA	**52** (31/60)	**48** (29/60)
QFA (200 µl)	**87** (105/121)	**13** (16/121)
QFA (70 µl)	**77** (40/52)	**23** (12/52)
QFA (20 µl)	**75** (41/55)	**25** (14/55)
Age matched females	**73** (22/30)	**27** (8/30)

* Figures in brackets = number of positive or collapsed islets/total number examined.

Example 4:

This example is designed to demonstrate the effectiveness of killed *Coxiella burnetti* treatment in preventing the recurrence of IDDM in spontaneously diabetic NOD mice transplanted with syngeneic islet tissue.

Donor animals are anesthetised with an ip injection of Avertin solution. A curved 27g needle is inserted into the common bile duct at the hilus after the distal end of the duct has been clamped. Approximately 3 ml of cold Collagenase P (Boehringer Mannheim, Castle Hill, NSW) made to 2.5 mg/ml HBSS + 0.05% BSA is injected immediately after dissection of the intrathoracic aorta. The pancreas is then excised and digested while held stationary in a 37°C water bath for 15 minutes and islets are then hand-picked and transplanted essentially as described in Bowen et al. (Bowen *et al.*, 1980).

Transplanted mice are divided into control and killed *Coxiella burnetti* treatment groups. Treatment groups are given killed *Coxiella burnetti* either at the time of islet transfer (Group 1) or 24 hours after islet transplantation (Group 2). Control islet recipient mice receive saline instead of killed *Coxiella burnetti*. All islet-engrafted mice are monitored for the presence of disease by measuring glucose levels in the urine and if elevated levels of glucose are found, hyperglycaemia is confirmed by measuring levels of glucose in the blood. Killed *Coxiella burnetti* treated, islet-transplanted mice will not develop hyperglycaemia, while the saline treated islet recipients will develop hyperglycaemia within two weeks of islet transplantation.

Example 5:

This example demonstrates that *Coxiella burnetti* vaccine (QVAX) is effective in inhibiting the occurrence of IDDM in NOD mice.

Female NOD littermates 72 days of age were divided into two groups and injected subcutaneously with either 100 µl of QVAX (obtained from CSL,

Melbourne, Australia) or 100 μl of saline solution. Ten mice were treated with QVAX while 10 littermates received saline solution.

Mice in both groups were checked three times per week (Mon., Wed. and Fri.) for elevated glucose concentrations in their urine (Tes-Tape, Eli Lilly and Co., Indianapolis, IN). When mice were found to have raised urinary glucose levels, whole blood glucose levels were determined with a Companion 2 Sensor (Medisense, Cambridge, Mass.). Where two consecutive daily blood glucose readings above the 95% confidence interval for NOD mice in this colony (3.9-9.1 mmol/L) were obtained, the animal was deemed to be diabetic. Animals were monitored until they reached 100 days of age or became diabetic.

None of the QVAX treated mice became diabetic during this period while 2 out of 10 of the saline treated mice became diabetic by 100 days of age.

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The Australian National University

By its Patent Attorneys
Davies Collison Cave

Figure 1 QFA Rx Prevents IDDM

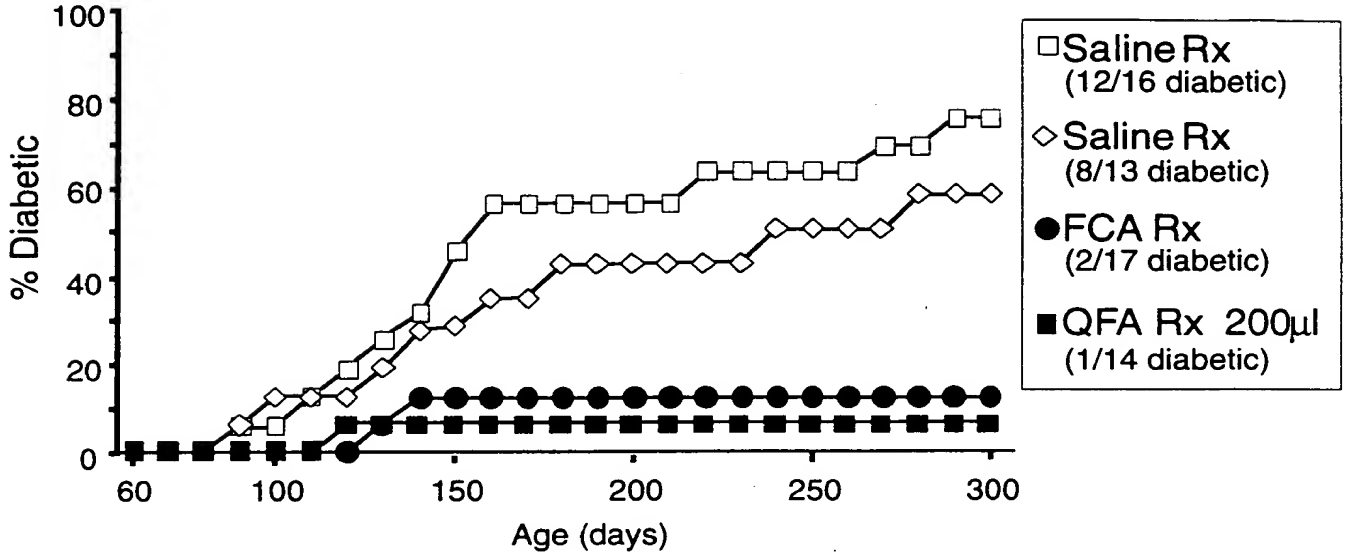

Figure 2 QFA 20 or 70 µl Rx Prevents IDDM